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Phylogenetic position of *Odorrana macrotympana* (Yang, 2008) (Anura, Ranidae) and extension of its geographical distribution

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Abstract

Based on a 16S rRNA gene fragment, a molecular phylogeny for the genus *Odorrana* Fei, Ye & Huang, 1990 was reconstructed, the validity of the poorly-known ranid species *O. macrotympana* (Yang, 2008) was confirmed and its phylogenetic position was evaluated. In addition, we report the first country record of *O. macrotympana* from Myanmar, based on our new records from Htamanthi Wildlife Sanctuary, Sagaing Division and present a supplementary description of this species. This report also constitutes the first record of *O. macrotympana* from outside of China.

Key Words

16S rRNA, China, Htamanthi Wildlife Sanctuary, morphology, Myanmar, new record, western Yunnan

Introduction

Odorrana macrotympana is a poorly-known ranid species described, based on only two specimens from Xueli Village, Taiping Town, Yingjiang County, Dehong Prefecture, Yunnan Province, China, close to the border with Myanmar (Yang and Rao 2008). This species was originally placed in the genus *Rana* Linnaeus, 1758 and was transferred into the genus *Odorrana* by Frost (2009).

Odorrana macrotympana most closely resembles *O. tiannanensis* (Yang & Li, 1980), but it is characterised by the following characters: lips uniform white without any spots or streaks, dorsal skin smooth and intermittent dorsolateral folds present, tympanum large and external vocal sac present in males (Yang and Rao 2008). *Odorrana macrotympana* was previously known only from the type locality and there have been few records of this species since its original description (Fei et al. 2009, 2012; AmphibiaChina 2021; Frost 2021).

As no molecular data were available for *Odorrana macrotympana* so far, the phylogenetic position of *O. macrotympana* has never been inferred and, thus, its

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systematic affinities remain unresolved. During our field surveys in Yunnan Province, China and in northern Myanmar from 2009 to 2021, we collected one topotype of *O. macrotympana* from its type locality, Yingjiang County, Dehong Prefecture, Yunnan Province, China; five specimens of *Odorrana* cf. *macrotympana* from Htamanthi Wildlife Sanctuary, northern Myanmar; and four topotypes of *O. tiannanensis* from its type locality, Hekou County, Honghe Prefecture, Yunnan Province, China, respectively. We collected DNA sequence data of these specimens in order to reconstruct a molecular phylogeny and evaluate the phylogenetic position of *O. macrotympana*.

In addition, our phylogenetic analysis of molecular data demonstrated that the specimens from northern Myanmar should be assigned to *Odorrana macrotympana*. Thus, this report constitutes the first record for the species outside China and from the country of Myanmar.

Materials and methods

Taxon sampling

Field survey in Yingjiang County, Yunnan Province, China, was conducted under permits from Tongbiguan Provincial Natural Reserve Management and Protection Bureau; field survey in Hekou County, Yunnan Province, China, was conducted under permits from Honghe Prefecture Forestry and Grassland Bureau of Yunnan Province; and the field survey in northern Myanmar was undertaken at the invitation of the Republic of the Union of Myanmar, Ministry of Natural Resources and Environmental Conservation, Forest Department, Forest Research Institute. After live photographs were taken, specimens were collected, euthanised with ethyl acetate and fixed in 75% ethanol for storage. Liver tissue samples were preserved in 99% ethanol for molecular analysis. The specimens from China were deposited in Kunming Institute of Zoology, Chinese Academy of Sciences (abbreviation: KIZ; address: Kunming, Yunnan, China) and specimens from Myanmar were deposited in Southeast Asia Biodiversity Research Institute, Chinese Academy of Sciences (abbreviation: SEABRI; address: Yezin, Nay Pyi Taw, Myanmar).

Morphology

Measurements were taken with a digital caliper to the nearest 0.1 mm by Shuo Liu. We measured: snout-vent length (SVL, from tip of snout to vent); head length (HL, from tip of snout to rear of jaw); head width (HW, width of head at widest point); snout length (SL, from tip of snout to anterior border of eye); eye diameter (ED, diameter of exposed portion of eyeball); tympanum diameter (TD, maximum diameter of tympanum); forearm and hand length (FHL, from elbow to tip of third finger); hand length (HAL, from the base of the outer palmar tubercle to the tip of third finger); total leg length (LEG, from vent to tip of fourth toe); tibia length (TL, distance from knee to heel); and tarsus and foot length (TFL, from heel to the tip of fourth toe). Morphological data of the type specimens of *Odorrana macrotympana* were taken from Yang and Rao (2008).

Collection and analysis of molecular data

Total genomic DNA was extracted from liver tissues using the DNeasy Tissue Kit (Qiagen, Inc., Valencia, CA). A fragment encoding mitochondrial 16S rRNA gene was amplified and sequenced. All new sequences have been deposited in GenBank, other sequences being downloaded from GenBank (Table 1). Sequences of *Rana chensinensis* David, 1875 and *Pelophylax nigromaculatus* (Hallowell, 1861) were used as outgroups (Chen et al. 2013; Feng et al. 2017; Yuan et al. 2019; Liu et al. 2021; Zhang et al. 2021). The primers L2188 (Matsui et al. 2006): 5'–AAAGTGGGCCTAAAAGCAGCCA–3' and 16H1 (Hedges 1994): 5'–CTCCGGTCTGAACTCAGATCACGTAGG–3' were used in amplification and cycle sequencing. Amplified DNA was produced in 25 µl reactions after 30 cycles of denaturation for 60 sec at 94 °C, annealing for 60 sec at 43–46 °C and extension for 1 min at 72 °C. PCR products were isolated through 1% agarose gel electrophoresis and further purified using Millipore Microcon Kits. Purified PCR products were sequenced by Davis Sequencing and sequences were edited and manually managed using SeqMan in Lasergene 7.1 (DNASTAR Inc., Madison, WI, USA) and MEGA X (Kumar et al. 2018).

Sequences were aligned using MAFFT 7 (Katoh and Standley 2013) with default parameters. The best substitution models were selected using the Bayesian Information Criterion (BIC) in ModelFinder (Kalyaanamoorthy et al. 2017) as implemented in IQ-TREE 1.6.12 (Nguyen et al. 2015). Bayesian Inference (BI) was performed in MrBayes 3.2.7 (Ronquist et al. 2012), based on the GTR+F+I+G4 substitution model and the Markov chains were run for 10,000,000 generations and sampled every 1,000 generations. Maximum Likelihood (ML) analysis was performed in IQ-TREE 1.6.12 (Nguyen et al. 2015), based on the TIM2+F+R4 substitution model and 1,000 bootstrap pseudoreplicates via the ultrafast bootstrap approximation algorithm were used to construct a final consensus tree. The genetic divergences (uncorrected p-distance) were calculated in MEGA X (Kumar et al. 2018).

Results

Phylogenetic analyses

The sequence alignment is 1095 bp in length. Topologies resulting from BI and ML analyses were consistent. The sequences of the specimens from Myanmar clustered with the sequence of our single topotype of *Odorrana macrotympana*; together, they represent a distinct lineage in the genus *Odorrana*, which is sister to *O. tiannanensis* with strong support (Fig. 1).

Table 1. Samples used for phylogenetic analyses of molecular sequence data. * = type locality.

Species	Locality	Voucher No.	GenBank No.
Odorrana absita	Xe Sap, Xe Kong, Laos*	FMNH258109	EU861542
Odorrana amamiensis	Tokunoshima, Ryukyu, Japan	KUHE24635	AB200947
Odorrana anlungensis	Anlong, Guizhou, China*	HNNU1008I109	KF185049
Odorrana aureola	Phu Luang, Loei, Thailand*	ZMKU AM 01137	KT002162
Odorrana bacboensis	Khe Moi, Nghe An, Vietnam*	FMNH255611	DQ650569
Odorrana banaorum	Tram Lap, Gia Lai, Vietnam	ROM7472	AF206487
Odorrana chapaensis	Lai Chau, Vietnam	AMNH A161439	DQ283372
Odorrana chloronota	Darjeeling, West Bengal, India*	BMNH 1947.2.28.6	DQ650594
Odorrana daorum	Sa Pa, Lao Cai, Vietnam	ROM19053	AF206482
Odorrana dulongensis	Dulongjiang, Yunnan, China*	KIZ035027	MW128102
Odorrana exiliversabilis	Wuyishan, Fujian, China*	HNNU0607032	KF185056
Odorrana fengkaiensis	Shiwanshan, Guangxi, China	HNNU295 7k	KF185033
Odorrana geminata	Cao Bo, Ha Giang, Vietnam	AMNH 163782	EU861546
Odorrana grahami	Kunming, Yunnan, China*	HNNU1008II016	KF185051
Odorrana graminea	Wuzhishan, Hainan, China*	HNNU0606123	KF185038
Odorrana hainanensis	Wuzhishan, Hainan, China*	HNNU0606105	KF185032
Odorrana hejiangensis	Hejiang, Sichuan, China*	HNNU1007I202	KF185052
Odorrana hosii	Kuala Lumpur, Malaysia	IABHU 21004	AB511284
Odorrana huanggangensis	Wuyishan, Fujian, China*	HNNU0607001	KF185059
Odorrana ishikawae	Amami Island, Ryukyu, Japan	No voucher	AB511282
Odorrana jingdongensis	Jingdong, Yunan, China*	20070711017	KF185050
Odorrana junlianensis	Junlian, Sichuan, China*	HNNU002	KF185058
Odorrana kuangwuensis	Nanjiang, Sichuan, China*	HNNU0908II185	KF185034
Odorrana kweichowensis	Shilian, Meitan, Guizhou, China	CIBGYU20130917004	MH193548
Odorrana leporipes	Shaoguan, Guangdong, China*	HNNU1008I099	KF185036
Odorrana lipuensis	Lipu, Guilin, Guangxi, China*	NHMG1306002	KM388699
Odorrana livida	Thagata Juwa, Myanmar*	BMNH 1889.2.25.48	DQ650615
Odorrana lungshengensis	Longsheng, Guangxi, China*	HNNU70028	KF185054
Odorrana macrotympana	Yingjiang, Yunnan, China*	KIZ 2009051020	OL831010
Odorrana macrotympana	Htamanthi, Sagaing, Myanmar	SEABRI 2019120040	OL831011
Odorrana macrotympana	Htamanthi, Sagaing, Myanmar	SEABRI 2019120041	OL831012
Odorrana macrotympana	Htamanthi, Sagaing, Myanmar	SEABRI 2019120072	OL831013
Odorrana macrotympana	Htamanthi, Sagaing, Myanmar	SEABRI 2019120073	OL831014
Odorrana margaretae	Emei, Sichuan, China	HNNU20050032	KF185035
Odorrana morafkai	Tram Lap, Gia Lai, Vietnam	ROM7446	AF206484
Odorrana nanjiangensis	Nanjiang, Sichuan, China*	HNNU1007I291	KF185042
Odorrana narina	Okinawa Island, Ryukyu, Japan	No voucher	AB511287
Odorrana nasica	Ha Tinh, Vietnam	AMNH A161169	DQ283345
Odorrana nasuta	Wuzhishan, Hainan, China*	HNNU051119	KF185053
Odorrana sangzhiensis	Sangzhi, Hunan, China*	CSUFT 4305220051	MW464865
Odorrana schmackeri	Yichang, Hubei, China*	HNNU0908II349	KF185047
Odorrana supranarina	Iriomotejima, Ryukyu, Japan	KUHE2898	AB200950
Odorrana swinhoana	Nantou, Taiwan, China	HNNUTW9	KF185046
Odorrana tianmuii	Tianmushan, Zhejiang, China*	NHMG1303018	KT315390
Odorrana tiannanensis	Hekou, Yunnan, China*	KIZ20193272	OL831009
Odorrana tiannanensis	Hekou, Yunnan, China*	KIZ20193273	OL831008
Odorrana tiannanensis	Hekou, Yunnan, China*	KIZ20193274	OL831007
Odorrana tiannanensis	Hekou, Yunnan, China*	KIZ20215191	OL831006
Odorrana tiannanensis	Hekou, Yunnan, China*	SCUM50510CHX	EF453751
Odorrana tormota	Huangshan, Anhui, China*	No voucher	DQ835616
Odorrana trankieni	Ta Xua, Son La, Vietnam*	VNMN04035	KX893900
Odorrana utsunomiyaorum	Iriomotejima, Ryukyu, Japan	KUHE12896	AB200952
Odorrana versabilis	Leishan, Guizhou, China*	HNNU003 LS	KF185055
Odorrana wuchuanensis	Wuchuan, Guizhou, China*	HNNU019 L	KF185043
Odorrana yentuensis	Tay Yen Tu, Bac Giang, Vietnam*	IEBRA.2015.38	KX893891
Odorrana yizhangensis	Yizhang, Hunan, China*	HNNU1008I075	KF185048
Odorrana yunnanensis	Longchuan, Yunnan, China*	HNNU001YN	KF185057
Pelophylax nigromaculatus	Aichi, Japan	No voucher	LC389208
Rana chensinensis	Ningshan, Shanxi, China	HNNU20060359	KF185061

Genetic distances

Genetic divergence (uncorrected p-distance) between the specimens from Myanmar and the topotype of *Odorrana macrotympana* was only 0.43% (Suppl. material 1: Table S1). Therefore, we considered the Myanmar population to be conspecific with *O. macrotympana*. Genetic divergence (uncorrected p-distance) between *O. macrotympana* and other species of *Odorrana* ranged from 9.51% to 16.87% (Suppl. material 1: Table S1), suggesting that *O. macrotympana* is a valid species.

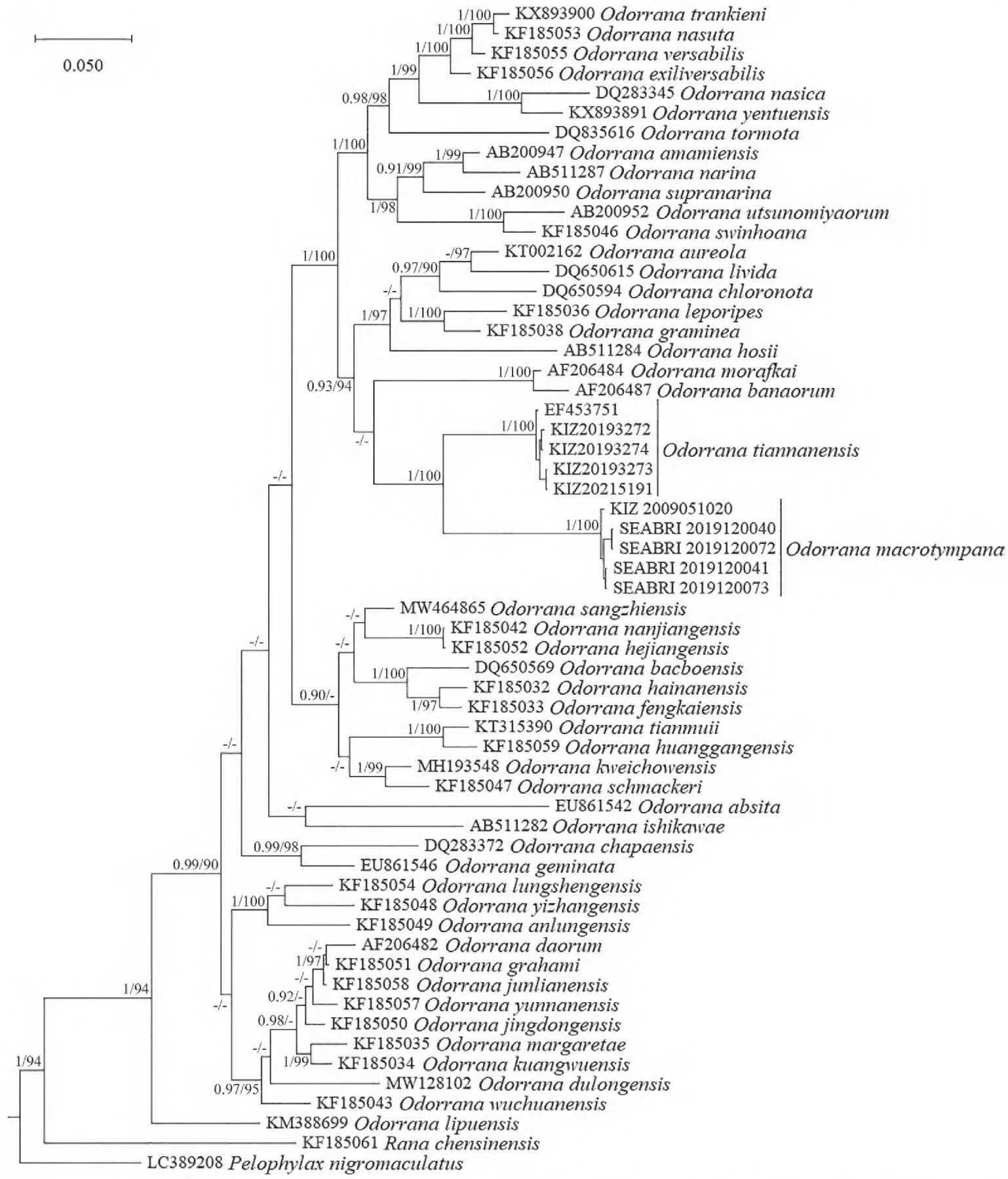

Figure 1. Phylogenetic tree of *Odorrana*, inferred from BI analysis of a 1095 bp fragment of the 16S rRNA. Numbers before slashes indicate Bayesian posterior probabilities (≥ 0.90) and numbers after slashes indicate bootstrap support from a separate ML analysis (≥ 90).

Taxonomic accounts

Odorrana macrotympana (Yang, 2008)

Suggested common English name "Large Eared Odorous Frog"

Figs 2–6 and 8B

Holotype. KIZ 94001, adult female.

Paratype. KIZ 94002, adult male.

Type locality. Xueli Village, Taiping Town, Yingjiang County, Dehong Prefecture, Yunnan Province, China.

Other materials. KIZ 2009051020, One adult female, collected by Dingqi Rao from Xueli Village, Taiping Town, Yingjiang County, Dehong Prefecture, Yunnan Province, China (24°26'29"N, 97°33'42"E, 330 m elevation), on 10 May 2009. SEABRI 2019120040, one adult male and SEABRI 2019120041, one adult female, collected by Shuo Liu from Nam Pa Gon section, Htamanthi Wildlife Sanctuary, Sagaing Division, Myanmar (25°19'3"N, 95°31'43"E, 140 m elevation), on 2 December 2019. SEABRI 2019120072 and SEABRI 2019120074, two adult males and SEABRI 2019120073, one adult female, collected by Shuo Liu from Nam E Zu section, Htamanthi Wildlife Sanctuary, Sagaing Division, Myanmar (25°28'8"N, 95°38'57"E, 130 m elevation), on 9 December 2019.

Description of the specimens from Myanmar. Measurements and the comparison with the type specimens are represented in Table 2. Habitus moderately slender, SVL 56.7–59.1 mm in males and 91.6–111.2 mm in females; head width slightly shorter than length (HW/HL 0.89–0.97); snout obtuse, pointed in dorsal view and rounded in profile, obviously projecting beyond lower jaw; position of nostril dorsolateral, closer to snout than

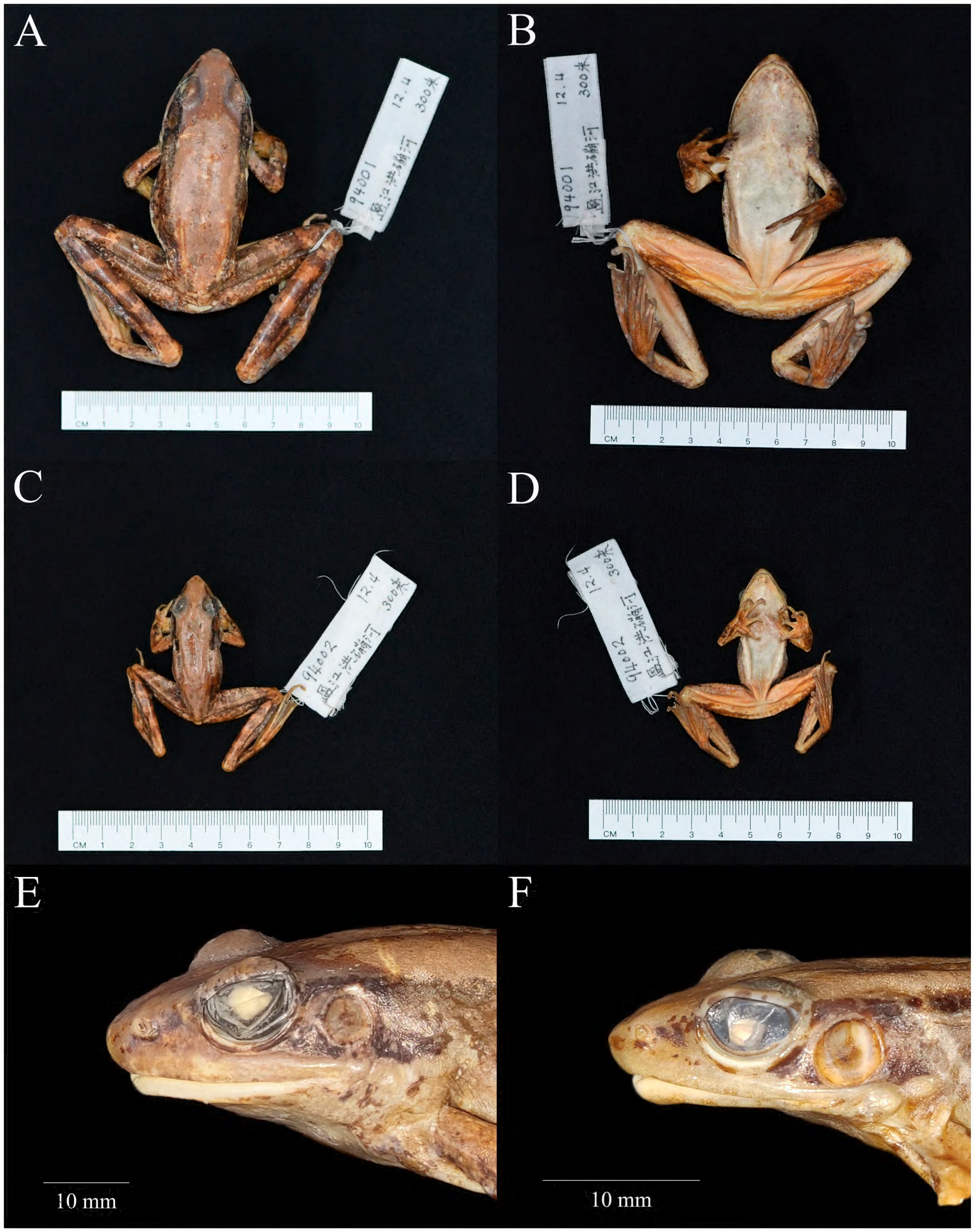

Figure 2. Type specimens of *Odorrana macrotympana* in preservative: **A.** Dorsal and **B.** Ventral, view of the holotype (KIZ 94001, female); **C.** Dorsal and **D.** Ventral, view of the paratype (KIZ 94002, male); **E.** Lateral view of the head of the holotype (KIZ 94001, female); **F.** Lateral view of the head of the paratype (KIZ 94002, male). Photo by Shuo Liu.

eye; canthus rostralis distinct; loreal region concave and vertical; internarial distance greater than interorbital distance; snout length greater than eye diameter; pineal body distinct; supratympanic fold horizontal and straight; tympanum very large in males (TD/ED 0.71–0.78) and relatively small in females (TD/ED 0.56–0.62), round and transparent; vomerine teeth distinct; choanae close to the vomerine teeth. Tongue cordiform, posterior notch

Table 2. Measurements (mm) of *Odorrana macrotympana*. See text for character definitions. Data of the type specimens were obtained from the original description (Yang and Rao 2008). Notes: HAL of holotype, originally reported as "26 mm" is parenthetically included, along with the true value (16 mm).

	KIZ 94001	KIZ 94002	SEABRI	SEABRI	SEABRI	SEABRI	SEABRI
	Holotype	Paratype	2019120040	2019120041	2019120072	2019120073	2019120074
	Female	Male	Male	Female	Male	Female	Male
SVL	95	50	56.8	111.2	59.1	91.6	56.7
HL	35.5	20	22.3	40.6	24.8	33.4	23.8
HW	32	17	21.2	37.2	22.0	32.5	22.2
SL	15	8	10.1	18.2	10.3	15.6	10.8
ED	13	7.5	7.9	11.7	8.2	9.2	8.0
TD	6	5.5	5.6	6.6	5.8	5.7	6.2
FHL	41.5	24	28.5	50.6	29.7	45.2	27.2
HAL	16 (26)	15.5	16.4	27.7	18.6	26.5	17.9
LEG	185	100	117.0	219.0	121.0	195.5	117.0
TL	63.5	32.5	36.2	68.7	38.5	60.2	35.9
TFL	75.5	42.5	46.2	84.5	49.7	76.5	46.3

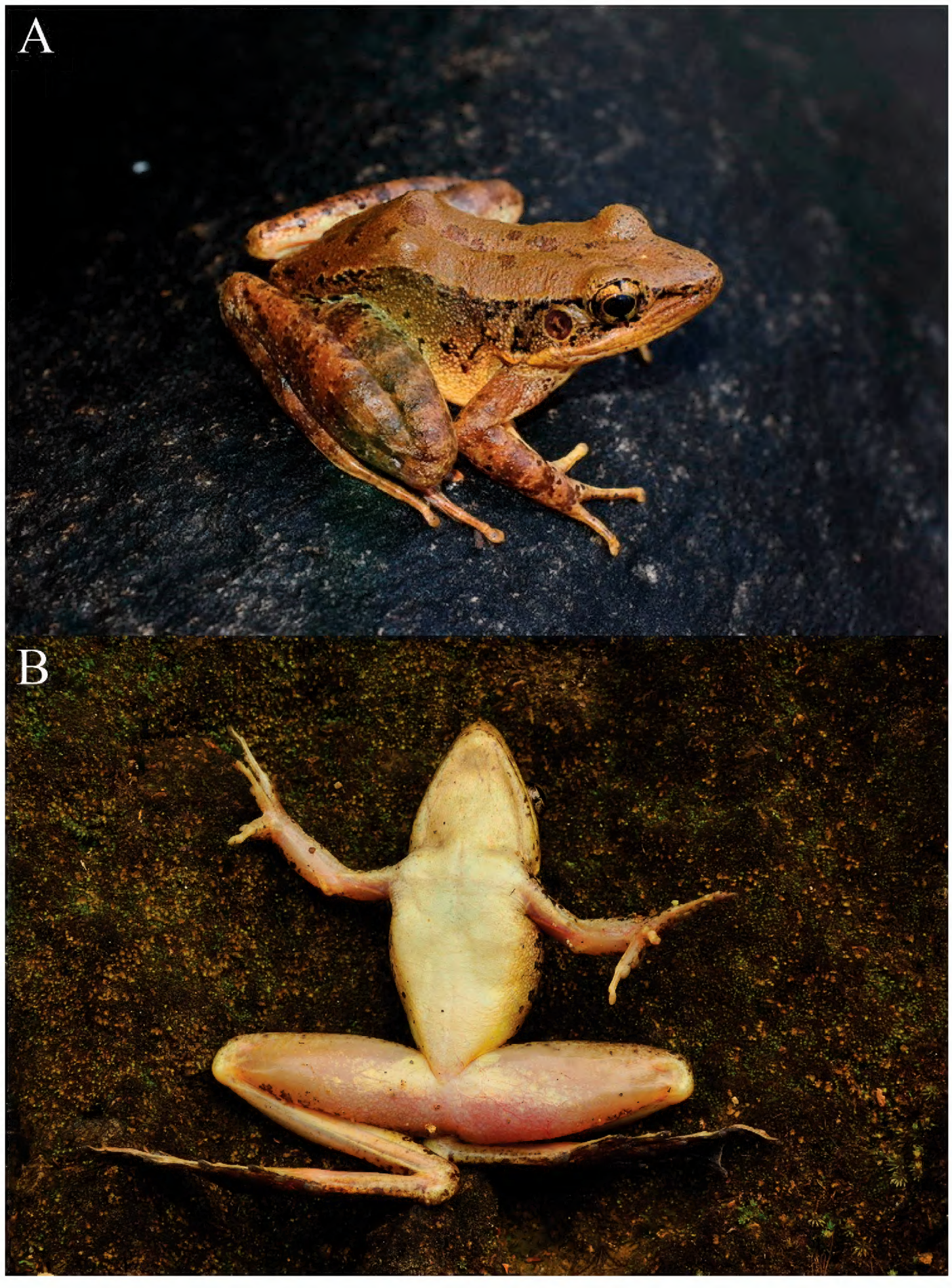

Figure 3. Adult female topotype of *Odorrana macrotympana* from its type locality: **A.** Dorsal view; **B.** Ventral view. Photo by Xiaofeng Ma and Hong Hui.

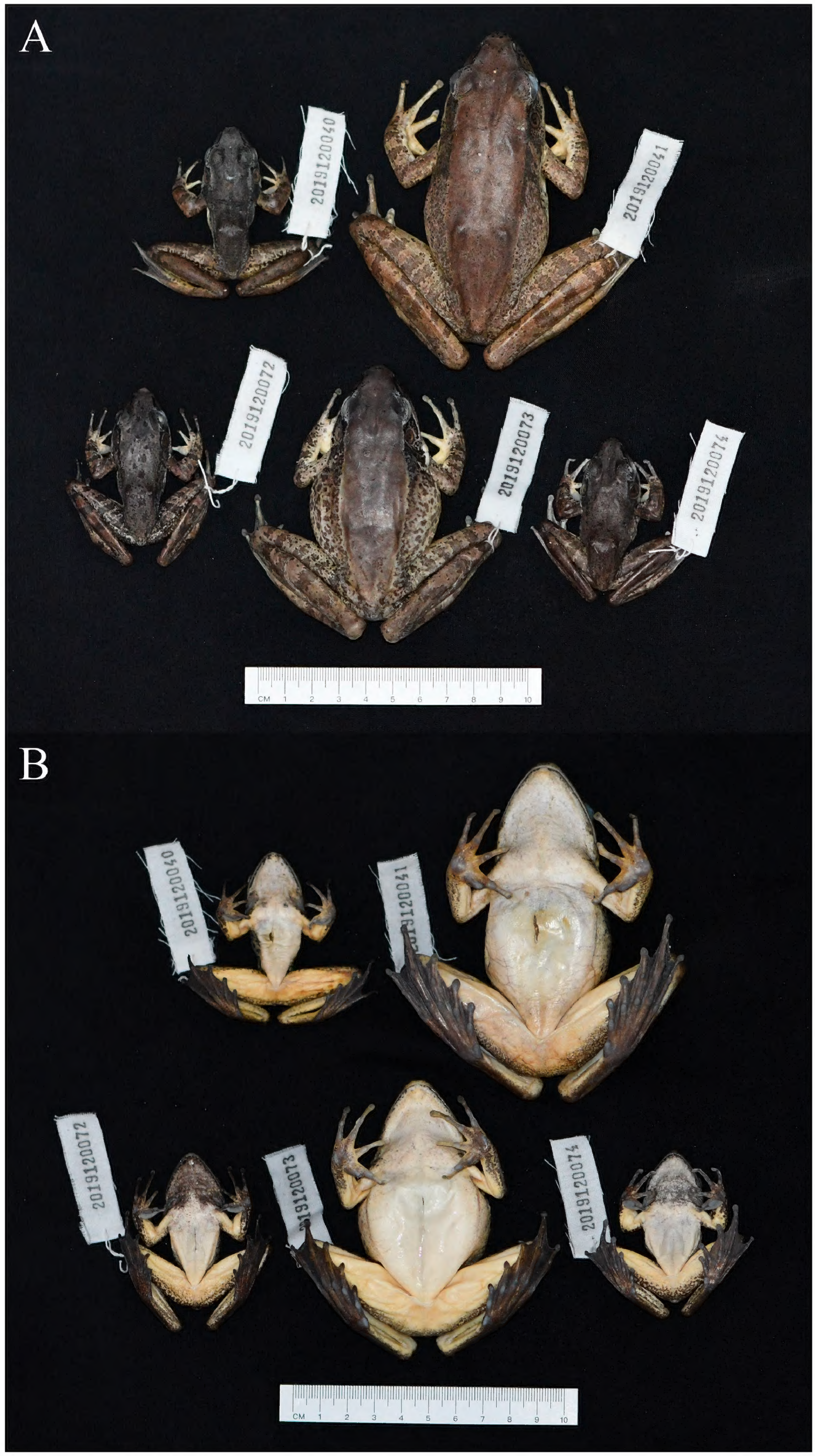

Figure 4. Specimens of *Odorrana macrotympana* from Myanmar in preservative: **A.** Dorsal view; **B.** Ventral view. Photo by Shuo Liu.

Figure 5. Live colour variation in *Odorrana macrotympana* from Htamanthi Wildlife Sanctuary, Sagaing Division, Myanmar: **A, B, C.** Adult males; **D.** Subadult female; **E, F.** Adult females. Photo by Shuo Liu.

enlarged and formed as U-shaped; vocal sac openings visible on floor of mouth, in each corner; paired external vocal sacs present in males.

Forelimbs robust in males and relatively weak in females; relative lengths of fingers III > IV > I > II; all finger tips slightly expanded; lateral fringes and webbing on fingers absent; subarticular tubercle round and prominent; inner metacarpal tubercle and outer metacarpal tubercle distinct; greyish-yellow glandular nuptial pad on finger I in males.

Hind limbs long, tibia slightly longer than thigh; toes long and thin, relative lengths IV > V > III > II > I; all toe tips slightly expanded; entire webbing; subarticular tubercles prominent and longitudinally ovoid; inner metatarsal tubercle prominent and oval; outer metatarsal absent.

Dorsal skin smooth, lateral skin granular, ventral skin smooth. Dorsolateral folds distinct.

Colouration in life. Dorsum greyish-brown, brownish-green or solid green with a few or many tiny black

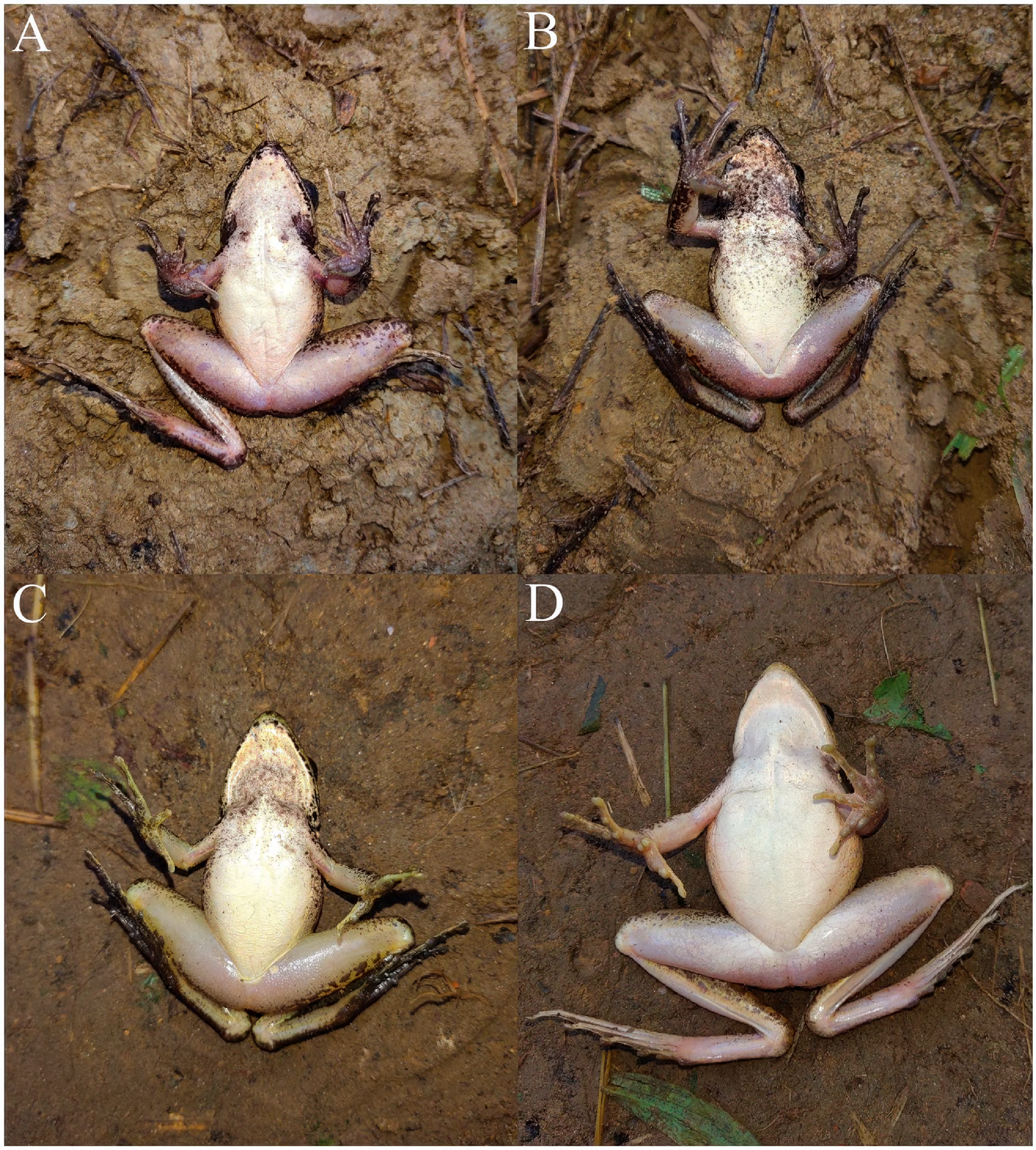

Figure 6. Live ventral colour variation in *Odorrana macrotympana* from Myanmar: **A, B.** Adult males; **C.** Subadult female; **D.** Adult female. Photo by Shuo Liu.

spots and several distinct or indistinct large black or brown spots. Upper and lower lips greyish-white, yellow, light green or brown with a few or many brown or black spots. Lateral side of head and body greyish-brown with many large or tiny black spots. Dorsal surfaces of limbs greyish-brown with some brown or black bands. Ventral surface of throat, chest and belly uniform white or some stains on ventral surface of throat, chest and anterior part of belly. Ventral surface of limbs white or pink. Tympanum brown, iris greyish-brown, pupil black.

Colouration in preservative. In preservative, dorsum fades to greyish-black. Ventral surface of throat, chest, belly and limbs pale white. Iris black, pupil white.

Sexual dimorphism. Body size much smaller in adult males than in adult females, adult males have relatively stouter forelimbs than females and greyish-yellow glandular nuptial pad on finger I, external vocal sacs present in adult males.

Natural history notes. In Myanmar, all specimens of *Odorrana macrotympana* were found on the banks of

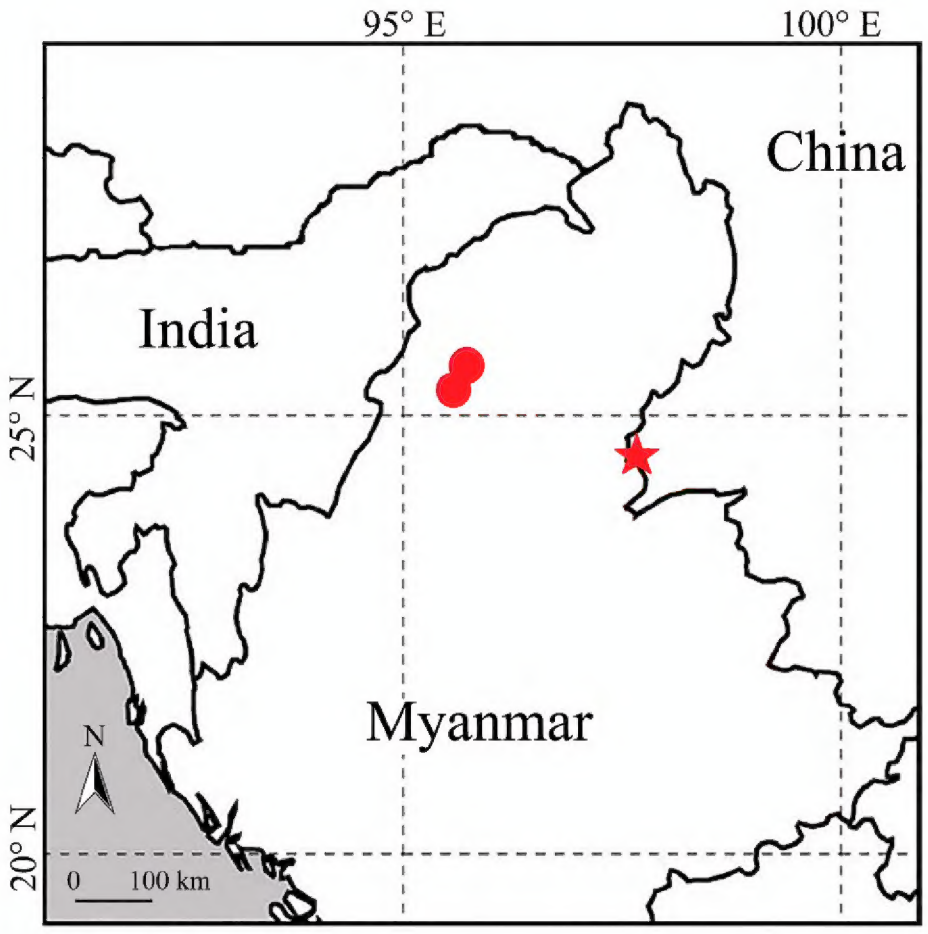

Figure 7. Type locality in western Yunnan, China (red star) and new collection sites in northern Myanmar (red dots) summarising all known localities for *Odorrana macrotympana*.

large rivers. During December, we heard the courtship calls from males on the river banks at night, the sound of the calls being similar to those of other *Odorrana* species. We also observed males and females in amplexus on the banks of the rivers. No eggs or tadpoles were found.

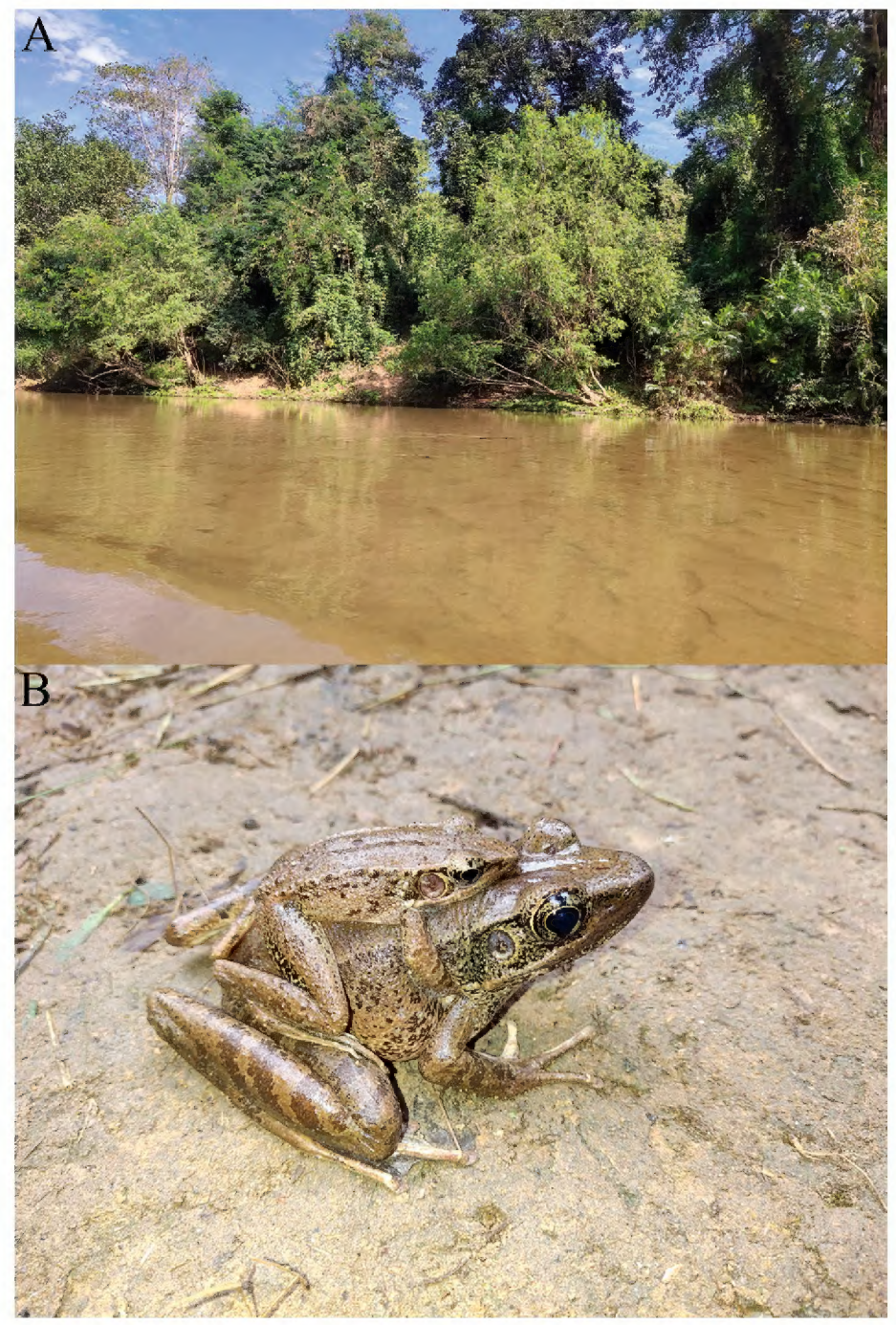

Figure 8. Habitat of *Odorrana macrotympana* in **A.** The Htamanthi Wildlife Sanctuary, Myanmar; **B.** Male and female in amplexus. Photo by Shuo Liu.

Discussion

According to Yang and Rao (2008), *Odorrana macrotympana* is a distinct species and most closely resembles *O. tiannanensis*. Our molecular analyses revealed that *O. macrotympana* was a sister lineage of *O. tiannanensis* (Fig. 1) and the genetic divergence between *O. macrotympana* and *O. tiannanensis* was 9.51% (Suppl. material 1: Table S1). This study agrees with Yang and Rao (2008) that *O. macrotympana* is a valid species and closely related to *O. tiannanensis*, although they are distributed far apart (the distance between the type localities of *O. macrotympana* and *O. tiannanensis* is approximately 660 km).

Previously, *Odorrana macrotympana* was known only from its type locality (Yang and Rao 2008; Fei et al. 2009, 2012; AmphibiaChina 2021; Frost 2021), located by the Dayingjiang River, a tributary of the Ayeyarwady River. Here, we report collections of this species from Htamanthi Wildlife Sanctuary, northern Myanmar, located by the Chindwin River, also a tributary of the Ayeyarwady River. Our finding significantly extended the known distribution of this species, indicating that this species is widely distributed in the Ayeyarwady River system. The new Myanmar record is approximately 230 km from the type locality in China. Additionally, within the Htamanthi Wildlife Sanctuary, Myanmar, *O. macrotympana* was quite abundant (we observed a large population at ~ 140 m elevation). However, at the type locality in China (~ 300 m elevation), the species is not abundant and the original population is comparatively small. We speculate that western Yunnan forms the edge of its distribution and northern Myanmar may be closer to the centre of this species' distribution.

Morphologically, the characters of the specimens from northern Myanmar mostly agree with the original description of *Odorrana macrotympana* apart from a few differences. According to the original description of *O. macrotympana*, the dorsum is greyish-brown and upper and lower lips are uniform white without any spots or streaks (Yang and Rao 2008). However, dorsal surfaces of specimens from northern Myanmar vary from greyish-brown to brownish-green or solid green and lips vary from greyish-white to yellow, light green or brown with variable dark spots. Thus, dorsal and labial colour characteristics, originally proposed as diagnostic characters for this species, may be more variable (and non-diagnostic) than originally reported (Yang and Rao 2008).

The type specimens of *Odorrana macrotympana* were collected in December 1994 and females have been observed to deposit eggs in February of the following year (Yang and Rao 2008). We heard male courtship calls and observed pairs in amplexus during December in northern Myanmar, which is basically consistent with original reports from China (Yang and Rao 2008). It is unknown

whether this species also reproduces during months outside of this (December–February) range.

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Supplementary material 1

Table S1

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Data type: Table (MS EXCEL file)

Explanation note: Genetic divergence (uncorrected p-distance) (%) based on the mitochondrial 16S rRNA gene sequences.

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